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Complete *cis* Exclusion upon Duplication of the Eμ Enhancer at the Immunoglobulin Heavy Chain Locus

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Developing lymphocytes somatically diversify their antigen-receptor loci through V(D)J recombination. The process is associated with allelic exclusion, which results in monoallelic expression of an antigen receptor locus. Various *cis*-regulatory elements control V(D)J recombination in a developmentally regulated manner, but their role in allelic exclusion is still unclear. At the immunoglobulin heavy chain locus (*IgH*), the Eμ enhancer plays a critical role in V(D)J recombination. We generated a mouse line with a replacement mutation in the constant region of the locus that duplicates the Eμ enhancer and allows premature expression of the γ3 heavy chain. Strikingly, IgM expression was completely and specifically excluded in *cis* from the mutant allele. This *cis* exclusion recapitulated the main features of allelic exclusion, including differential exclusion of variable genes. Notably, sense and antisense transcription within the distal variable domain and distal V_H-DJ_H recombination were inhibited. *cis* exclusion was established and stably maintained despite an active endogenous Eμ enhancer. The data reveal the importance of the dynamic, developmental stage-dependent interplay between *IgH* locus enhancers and signaling in the induction and maintenance of allelic exclusion.

Developing B and T lymphocytes have the capacity to somatically alter their genomes and diversify their antigen receptor loci through V(D)J recombination. This developmentally regulated process is initiated by the lymphoid-specific RAG1/2 complex, which recognizes conserved recombination signal sequences (RSSs) flanking the V, D, and J segments in the variable domain of antigen receptor (*IgH*, *IgL*, and *TCR*) loci (1, 2). V(D)J recombination correlates with chromatin modifications, germ line transcription of rearranging V, D, and J segments, and large-scale chromosome dynamics within nuclear compartments (3–5).

The mouse *IgH* locus contains ~200 V_H genes, which are subdivided into V_H gene families. The most prominent are the distal V_H genes, notably the large V_{HJ558} gene family, and the proximal V_H genes. These are followed by a dozen D segments (~60 kb), 4 J_H segments (~2 kb), and 8 constant genes (~200 kb) (6, 7).

In B lymphocytes, V(D)J assembly starts at the *IgH* locus, where D segments are first recombined to J_H segments on both alleles. Although they have the potential to undergo V_H-DJ_H recombination on the two alleles, the vast majority of B lymphocytes are subject to allelic exclusion, i.e., a given B cell expresses only one *IgH* allele. A productive V(D)J rearrangement allows production of the μ heavy chain, which associates with surrogate light chains and signals an arrest of V_H-DJ_H recombination on the second *IgH* allele. If the first V_H-DJ_H rearrangement is not productive, then the second allele can undergo V_H-DJ_H recombination (1, 8). Nonetheless, in rare B cells, productive rearrangements can occur on both alleles (i.e., allelic inclusion), but only one μ heavy chain from only one allele can associate with surrogate light chains (9).

Several lines of evidence support the notion that V_H-DJ_H rearrangement is the regulated step in *IgH* allelic exclusion and that allelic exclusion is maintained by a feedback mechanism (1, 8). However, while feedback inhibition of V_H-DJ_H recombination can explain the maintenance of allelic exclusion, the mechanisms that control its initiation are unknown, and various models have been proposed to account for the unequal availabilities of the two alleles for V_H recombination, including stochastic choice and differential epigenetic marks (1, 8, 10). Moreover, it remains unclear how *cis*-acting elements, which regulate V(D)J recombination in a cell type- and developmental stage-specific manner, control the suppression of V_H-DJ_H recombination during allelic exclusion.

In this context, various *cis*-regulatory elements, including enhancers and insulators, were identified at the *IgH* locus. The Eμ enhancer, located between the variable and constant domains, plays an important role in V(D)J recombination. Deletion or insulation of this element affects V(D)J recombination and sense and antisense transcription at specific sites of the *IgH* variable locus (11–14). The Eμ enhancer was also suggested to play an important role in allelic exclusion, as deletion of this element in a mouse model carrying a prerearranged V(D)J gene led to a substantial increase in B cell populations with allelic inclusion (15). Additionally, an "intergenic control region" (IGCR1) with insulator activity was identified between the V_H and D clusters (16–19). Deletion of CTCF sites within this region perturbed germ line transcription and recombination of the proximal V_H genes, the order and cell type specificity of V(D)J recombination, and feedback regulation and allelic exclusion of proximal V_H-DJ_H recombination (19, 20). Another major *cis*-acting element is the 3′ regulatory region (3′RR), composed of four

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enhancers lying downstream of the *IgH* locus (21). Targeted deletion studies showed that the 3′RR affected IgM expression in resting B cells (22, 23), but its role in allelic exclusion is unknown.

Mature B cells have the unique ability to undergo an additional recombination-mediated diversification process through class switch recombination (CSR), which specifically targets the constant genes of the *IgH* locus. At the genomic level, CSR occurs between highly repetitive switch (S) sequences, located upstream of the constant genes. CSR thus enables activated B cells to switch from the expression of IgM to the expression of downstream isotypes (IgG, IgE, or IgA) (24). Determining whether these isotypes can replace IgM in promoting B cell development and allelic exclusion was approached by using transgenic mice or mice with an engineered endogenous *IgH* locus which express IgG1 or IgA, but this led to conflicting results (e.g., see references 25 to 29; discussed in references 27 and 28).

In this study, we analyzed a mouse line with a duplicated Eμ enhancer in the context of IgG3-driven B cell development. We show that duplication of Eμ leads to a phenomenon that we term *cis* exclusion, as distinct from allelic exclusion, by which the μ heavy chain gene from the mutant allele is stably and specifically excluded in *cis*.

MATERIALS AND METHODS

Mice. The generation of the mutant mouse line is described in the supplemental material. B1-8 mice were provided by K. Rajewsky. The experiments on mice were carried out according to the CNRS ethical guidelines and were approved by the Regional (Midi-Pyrénées) Ethical Committee.

Flow cytometry analyses. Sample preparation, staining, and antibodies and their sources are described in the supplemental material.

V(D)J rearrangement assays. B cells from bone marrows were first sorted by using CD19-magnetic microbeads and LS columns (Miltenyi) and then labeled with anti-B220, anti-CD43, and anti-IgM, anti-IgG3, or anti-κ. The sorted pro-B (IgM^- $B220^+$ $CD43^{high}$ [wild type {WT}], $IgG3^-$ $B220^+$ $CD43^{high}$ [A150], or κ^- $B220^+$ $CD43^{high}$ [WT and A150]) and pre-B (IgM^- $B220^+$ $CD43^{low}$ [WT], $IgG3^-$ $B220^+$ $CD43^{low}$ [A150], or κ^- $B220^+$ $CD43^{low}$ [WT and A150]) cell fractions were harvested, and genomic DNAs were prepared by using a Puregene core kit A (Qiagen). Splenic B cells were negatively sorted by using CD43-magnetic microbeads and LS columns (Miltenyi). Genomic DNAs were resuspended and diluted for the PCR assay. The primers, normalization of amounts of DNA, and quantification were described previously (14, 30). Additional primers are listed in Table S3 in the supplemental material. The purity of the sorted populations was checked by fluorescence-activated cell sorter (FACS) analysis and by the rearrangement status of the κ locus.

Reverse transcription-PCR (RT-PCR). B cell precursors from $Rag2^{-/-}$ bone marrows were sorted using CD19-magnetic microbeads (Miltenyi). Pro-B and pre-B cells were sorted as described above. Bone marrow IgM^{b+} and $IgG3^+$ single expressers were sorted after staining with anti-B200, anti-IgM^b, and anti-IgG3. Total RNA was reverse transcribed (Invitrogen) and subjected to semiquantitative PCR or real-time PCR. The primers, normalization, and quantification were described previously (14, 30). Additional primers are listed in Table S3 in the supplemental material.

Statistical analysis. Results are expressed as means ± standard errors of the means (SEM) (GraphPad Prism), and overall differences between values from WT and mutant mice were evaluated by two-tailed unpaired Student's *t* test with Welch's correction. The difference between means is significant if the *P* value is <0.05, very significant if the *P* value is <0.01, and extremely significant if the *P* value is <0.001.

RESULTS

Replacement of the Iγ3 germ line promoter by a prerearranged V(D)J-Eμ cassette. We previously showed that replacement of the Iγ3 germ line promoter with the Iγ1 promoter insulated ectopic Iγ1 but not endogenous Iγ1 from the 3′RR, suggesting a preferential interaction of the 3′RR with the most proximal promoter during CSR (31). Recent studies detected stable interactions between Eμ and 3′RR in Rag-deficient pro-B cells (18, 19, 32, 33). If the 3′RR indeed cooperates with Eμ in early B cell development, then ectopic, 3′RR-proximal Eμ may similarly insulate the endogenous Eμ enhancer and potentially affect *IgH* recombination/expression and B cell development. We resorted to a replacement approach whereby the Iγ3 promoter was replaced by a V_HDJ_{H2}-J_{H3}-J_{H4}-Eμ unit, leaving intact the known *cis* elements required for V(D)J recombination and *IgH* expression and enabling premature expression of the γ3 heavy chain. The mutant line is called A150 henceforth (see Fig. S1 in the supplemental material).

B cell development under conditions of allelic inclusion, allelic exclusion, and isotypic competition. We first analyzed the effect of premature expression of IgG3 on B cell development in various compartments of homozygous mice by using FACS. A striking and constant finding was that IgM-expressing B cells were barely detectable (<1%) in every compartment analyzed, while IgG3-expressing B cells formed the vast majority of B cell populations (see Fig. S2 in the supplemental material).

The absence of IgM expression in A150/A150 mice, together with previous findings that B cells expressing class-switched isotypes were poor competitors of IgM-expressing B cells in heterozygotes (28, 29), led us to investigate the effect of the mutation on IgM expression. In the A150 model, this unfolds into three lines of inquiry: the effect of the mutation on IgM expression from the mutant allele, potential allelic inclusion, and allelic exclusion.

We used heterozygotes in which the WT allele was derived from a C57BL/6 mouse strain expressing the IgM^b allotype. As an additional control, we used the B1-8 mouse line, which allows premature expression of IgM^a and a nearly perfect allelic exclusion (34). In WT controls, IgM^{a+} and IgM^{b+} B cell populations were equally abundant in the bone marrow, whereas in B1-8/WT heterozygotes, the WT allele was completely excluded (Fig. 1A). Strikingly, surface IgM^a was totally absent in the bone marrow of A150/WT mice (Fig. 1A and C), indicating a perfect exclusion of the mutant allele in regard to IgM expression.

To investigate whether IgG3-expressing B cells can compete with IgM-expressing B cells, we looked at IgM and IgG3 surface expression patterns in the bone marrow of A150/WT and A150/B1-8 mice. In A150/WT mice, the WT allele must undergo productive V(D)J assembly in order to express IgM, whereas in A150/B1-8 mice, the B1-8-derived allele bears a prerearranged VDJ gene which enables premature expression of IgM (34). Hence, with these two models, we can correlate the isotypic competition status with allelic inclusion and/or V(D)J recombination.

The data show that B cells that prematurely express IgG3 can outcompete IgM-expressing B cells in A150/WT mice, since in the bone marrow, ~25% of B cells express exclusively IgG3, and ~11% express IgM only (Fig. 1B). In A150/B1-8 mice, in which both isotypes are prematurely expressed, the majority of B cells were subjected to allelic inclusion and coproduced IgM and IgG3 in the bone marrow and the spleen (Fig. 1B; see Fig. S2N in the supplemental material).

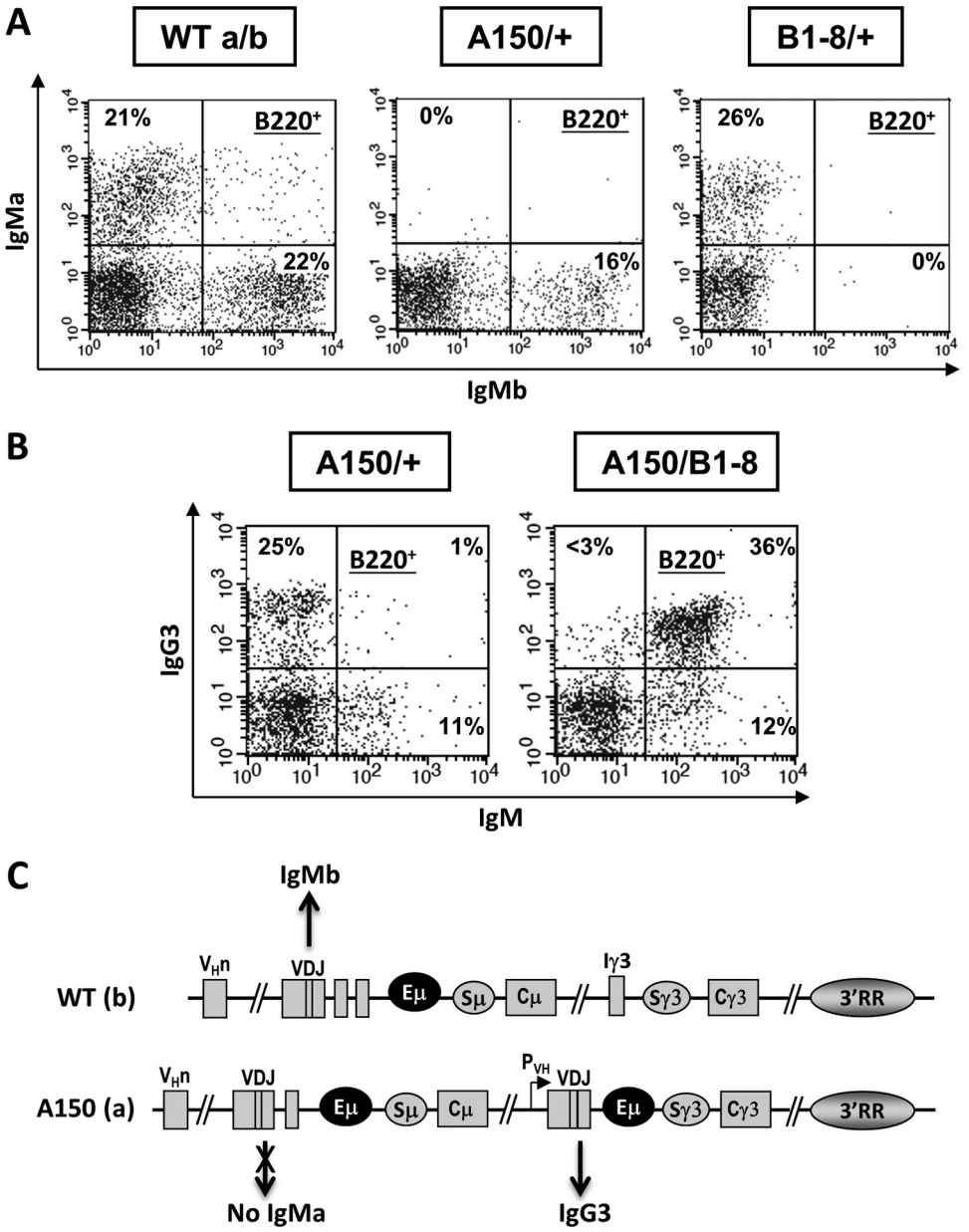

FIG 1 Flow cytometric analyses of allelic exclusion, allelic inclusion, and isotypic competition. (A) Single-cell suspensions from the bone marrows of mice with the indicated genotypes were stained with anti-B220 and monoclonal antibodies against the IgM^a and IgM^b allotypes and then gated on the $B220^+$ population. Representative plots are shown ($n = 5$). (B) Cells from the bone marrows of mice with the indicated genotypes were stained with anti-B220, anti-IgM, and anti-IgG3 and then gated on the $B220^+$ population. Representative plots are shown ($n = 4$). (C) Schematic recapitulating the FACS data for A150/+ mice. IgM is produced only from the wild-type allele. From the mutant allele, only IgG3 is expressed.

Interestingly, very few $IgG3^+$ IgM^+ double producers (<1%) were detected in the bone marrow of A150/WT mice, indicating that prematurely expressed IgG3 could mediate allelic exclusion (Fig. 1B) (see Discussion).

Together, the data show that the replacement mutation leads to a complete exclusion of IgM expression specifically from the mutant allele and that the prematurely expressed γ3 heavy chain can mediate robust allelic exclusion. In a context where the WT allele has to undergo V(D)J recombination, B cells that prematurely express IgG3 outcompete IgM-expressing B cells. Moreover, IgG3 and IgM can efficiently drive B cell development under conditions of allelic inclusion.

Reduction of proximal V_H-DJ_H recombination and inhibition of distal V_H-DJ_H recombination. The complete exclusion of IgM expression from the A150 mutant allele led us to suspect the involvement of mechanisms that act in *cis*, specifically the duplication of the Eμ enhancer, which may affect V(D)J recombination. In order to understand the molecular basis of *cis* exclusion, we performed a V(D)J recombination assay on sorted pro-B, pre-B, and splenic cells from homozygous A150 mice. This allowed us to investigate more precisely whether and at which step the mutation affects V(D)J assembly, independently of the V(D)J recombination events and allelic competition that occur in heterozygous mice.

We used a primer that pairs downstream of the J_{H4} segment

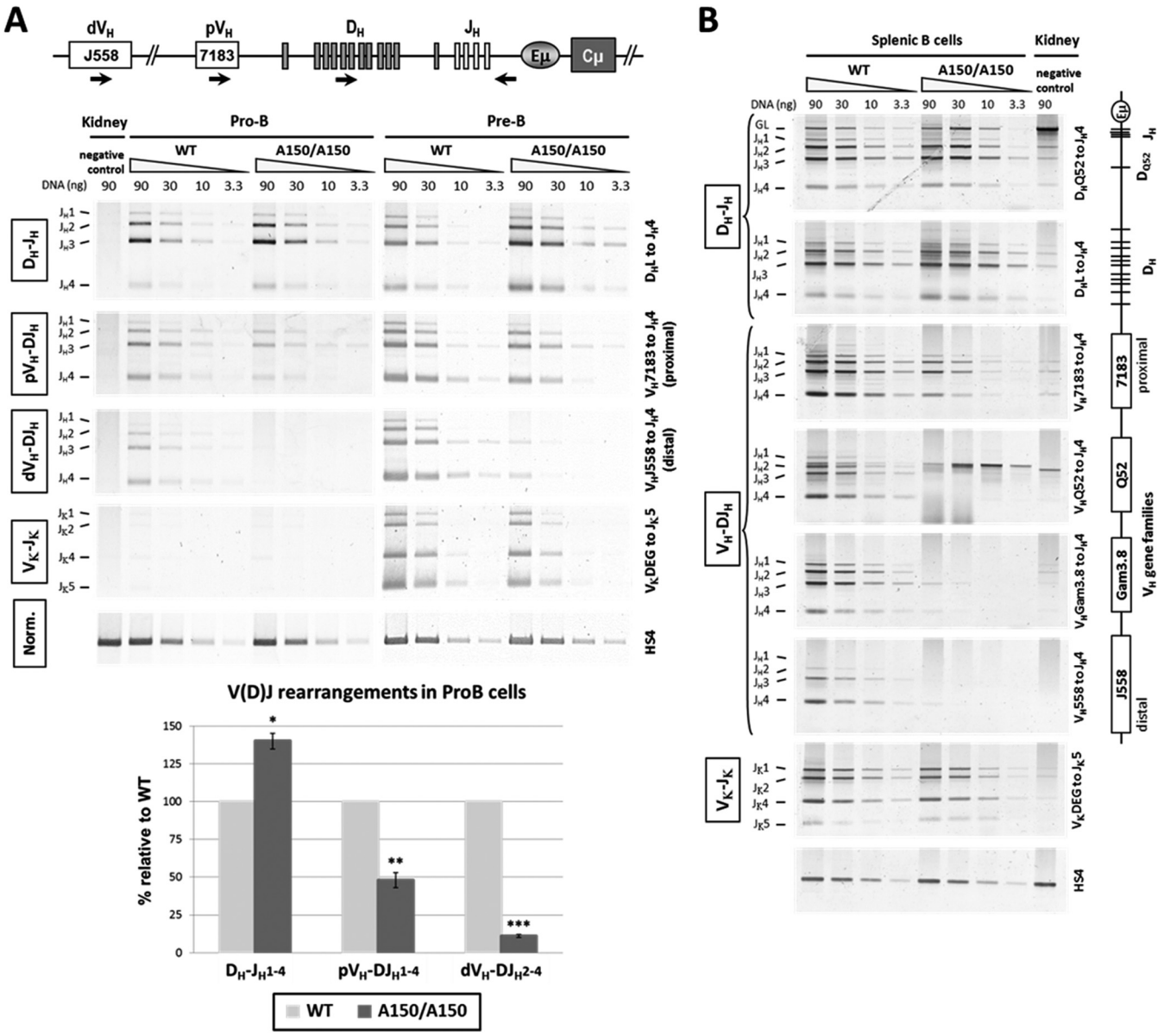

FIG 2 V(D)J rearrangement analyses. (A) Genomic DNAs were prepared from sorted pro-B and pre-B cells and were subjected to semiquantitative PCR to amplify D-J_H, V_H-DJ_H, and Vκ-Jκ rearrangements, using primers that bind the indicated segments and primers that pair 3′ of J_H4 (for the *IgH* locus) or 3′ of Jκ5 (for the κ locus). Kidney DNA was used as a negative control. A PCR analysis of the HS4 enhancer from the 3′RR was used for normalization of DNA input. The PCR for VκJκ rearrangement also allowed us to check the purity of the sorted populations ($n = 3$ for each experiment, with a pool of >5 mice). Quantification of the recombination events at the *IgH* locus of pro-B cells is displayed in the histogram. (B) Genomic DNAs were prepared from sorted splenic B cells and subjected to PCR as described for panel A ($n = 2$). The relative positions of the V_H gene families are indicated on the right. The signals detected for the V_{HQ52} gene segment in the A150 lanes were derived from the inserted V_H gene. Data are presented as means ± SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

and primers that bind different V_H and D gene segment families. We found a slight accumulation of DJ_H recombination intermediates in A150 pro-B cells. Interestingly, proximal V_H-DJ_H recombination was reduced and distal V_H-DJ_H recombination almost inhibited in pro-B cells, and only low levels of distal V_H-DJ_H recombination were detected in pre-B cells (Fig. 2A). Vκ-Jκ recombination was unaffected, indicating that the mutation did not interfere with the developmental stage-dependent retargeting of the RAG complex to the κ locus (Fig. 2A). In the spleen, while randomization of the repertoire was obvious in WT controls, V_H-DJ_H rearrangements involving V_H gene segment families located upstream of the proximal V_{H7183} family were essentially absent in A150 spleens (Fig. 2B).

Thus, the mutation leads to a mild accumulation of DJ_H alleles, a reduced amount of proximal V_H-DJ_H recombination, and a nearly complete inhibition of distal V_H-DJ_H recombination. We concluded that *cis* exclusion of IgM in A150 mice is mediated, at least in part, by reduced proximal V_H-DJ_H recombination and a profound impairment of distal V_H-DJ_H recombination.

Duplication of the Eμ enhancer severely affects distant germ line transcription. Given the impaired V_H-DJ_H recombination seen in A150 mice, and since sense and antisense germ line tran-

scription precedes V_H-DJ_H recombination (35, 36), we investigated the effect of Eμ duplication on germ line transcription. To this end, the A150 mutation was brought into the Rag2-deficient background, which precludes V(D)J recombination. Total RNA was extracted from bone marrow $CD19^+$ cells and analyzed by semiquantitative or real-time RT-PCR.

The data show that there were increased levels of the endogenous μ0 and Iμ sense transcripts, derived from the D_{Q52} promoter and the Eμ enhancer, respectively. In contrast, antisense transcripts across the D_H cluster were unaffected (Fig. 3A and B). Iμ-Cγ3 germ line transcripts and mature VDJ_{A150}-Cγ3 transcripts, derived from the ectopic Eμ and PV_H promoters, respectively, were readily detected in Rag2-deficient A150 pro-B cells (Fig. 3A and C). Interestingly, within the proximal V_H domain, both sense and antisense transcript levels were reduced but were readily detectable. In contrast, both sense and antisense transcripts were barely detectable within the distal V_H domain (Fig. 3D to G).

The data show that both noncoding and coding transcripts, derived from the ectopic Eμ enhancer and the PVH promoter, respectively, are already produced in Rag2-deficient pro-B cells. Importantly, the replacement mutation leads to a downregulation of germ line transcription within the proximal V_H domain and to an almost complete inhibition of germ line transcription within the distal V_H domain.

Reduced transcription of the endogenous μ heavy chain gene during *cis* exclusion. In order to investigate the potential transcriptional basis of IgM *cis* exclusion, we analyzed mature transcripts produced by the rearranged mutant allele. Total RNAs were prepared from sorted pro-B and pre-B cells and analyzed by semiquantitative RT-PCR.

By using primers that bind the leader sequence of the inserted V_H segment and the Cγ3-1 exon, γ3 transcripts were readily detected in A150 pro-B and pre-B cells (Fig. 4). With a Cμ1 reverse primer, μ transcripts were detected in WT pro-B and pre-B cells. In contrast, very low levels of these transcripts were detected in A150 pro-B and pre-B cells. Proximal V_H-containing γ3 transcripts were undetectable in WT controls, and only a faint signal was detected in A150 pre-B cells (Fig. 4). Interestingly, proximal V_H-containing μ transcripts were detected in both WT and A150 mice, but their levels were decreased ~10 times and ~5 times in mutant pro-B and pre-B cells, respectively (Fig. 4). Consistent with the inhibition of distal V_H-DJ_H recombination, distal V_H-containing μ transcripts were barely detectable (Fig. 4).

We concluded that *cis* exclusion of IgM correlates with a markedly decreased transcription of the endogenous proximal V_H-containing μ genes and with a virtual absence of distal V_H-containing μ gene expression.

Restored transcription of rearranged genes upon ectopic V(D)J recombination and deletion of the endogenous Eμ enhancer. Competition experiments showed that ~1/10 of heterozygous A150/WT B cells expressed IgM only (Fig. 1). This observation, together with the fact that IgM^a expression was totally excluded from the mutant allele and the γ3 heavy chain gene was already expressed in B cell precursors, led us to explore the mechanisms that switch off IgG3 production in the IgM^{b+} population. Among several possibilities (see Discussion and the supplemental material), we considered a deletional process that may involve either V(D)J recombination or CSR in pro-B/pre-B precursors of the IgM^{b+} population. Indeed, early transcription of the ectopic J_{H3} and J_{H4} RSSs and of Sγ3 (Fig. 3A) may provide accessible substrates for at least low levels of V(D)J recombination and Sμ/Sγ3 CSR, respectively. The data show that the loss of IgG3 expression in the IgM^{b+} population is due mainly to an ectopic V(D)J recombination which targets ectopic J_{H3} or J_{H4} RSSs (see Fig. S3 and S4 in the supplemental material).

Upon D-J_H recombination, Dμ transcripts derived from the promoter of the recombined D segment are produced (37, 38). We postulated that if ectopic D-J_H recombination occurred in pro-B precursors, one might detect Dγ3 transcripts in the IgM^{b+} population. While Dμ transcripts were detected in both populations, albeit at severely decreased levels, as expected, in the IgM^{b+} fraction, Dγ3 transcripts were detected exclusively in the IgM^{b+} population (Fig. 5), confirming the occurrence of ectopic D-J_H recombination on at least a fraction of A150 alleles and hinting at the possibility that another fraction had undergone ectopic V_H-DJ_H recombination. In this case, proximal V_H- and, potentially, distal V_H-containing γ3 transcripts should be detected.

In the $IgG3^+$ population, abundant mature VDJ_{A150}-Cγ3 transcripts were detected. In contrast, lower transcript levels were found in the IgM^{b+} population, indicating that a subset of this population has an intact VDJ_{A150} unit (Fig. 5; see Fig. S3 and S4 in the supplemental material). The proximal V_H- and distal V_H-containing μ transcripts were detected in both populations but were less abundant in the $IgG3^+$ population (Fig. 5). Interestingly, roughly equal levels of proximal V_H-containing γ3 transcripts were detected in the IgM^{b+} and $IgG3^+$ populations, suggesting that ectopic proximal V_H-DJ_H recombination and expression occurred on subsets of A150 alleles of the two populations (Fig. 5). Strikingly, while very low levels of distal V_H-containing γ3 transcripts were detected in A150/A150 controls (Fig. 4 and 5), they were now readily detectable in both the IgM^{b+} and $IgG3^+$ populations, indicating that a fraction of these populations had undergone ectopic distal V_H-DJ_H recombination and accumulated higher levels of μ transcripts.

Cloning and sequencing of proximal V_H- and distal V_H-containing γ3 cDNAs from the $IgG3^+$ population revealed an overwhelming majority (>95%) of productive rearrangements. In contrast, the majority of proximal V_H-containing γ3 (~59%) and distal V_H-containing γ3 (~76%) cDNA sequences from the IgM^{b+} population revealed nonproductive rearrangements. Within the limits of our data set, no evidence for recombination with cryptic RSSs was found (see Fig. S5 and Table S1 in the supplemental material).

The data show that the loss of IgG3 expression in the relatively small IgM^{b+} population of A150/WT bone marrow results mainly from an ectopic V(D)J recombination on the A150 allele. Importantly, the transcripts that were severely decreased (proximal V_H-containing γ3 transcripts) or virtually inhibited (distal V_H-containing γ3 transcripts) in homozygous A150 B cells appeared to be restored upon ectopic V(D)J recombination and associated deletion of endogenous Eμ.

DISCUSSION

A striking finding of this study is that the replacement mutation leads to a complete and stable *cis* exclusion of the μ gene specifically on the mutant allele. This pattern arose during ontogeny, was also established in the bone marrow, and was maintained in the lymphoid compartments of the adult mouse. In no instance did we detect IgM expression, or IgM and IgG3 coexpression, from

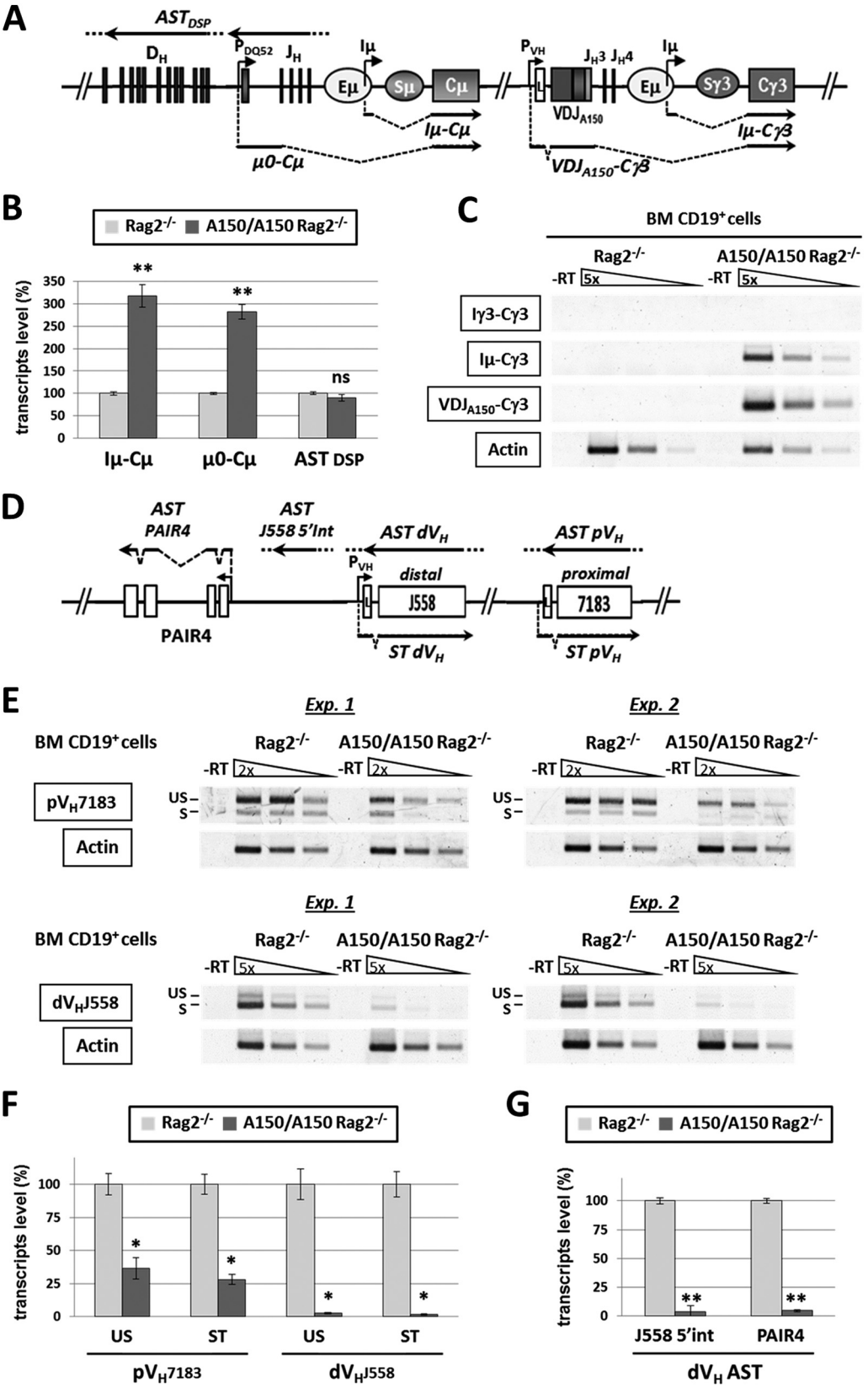

FIG 3 Analysis of germ line transcription in A150 B cell precursors. (A) Schematic showing the germ line and V(D)J_{A150} transcripts analyzed at the D-Cμ domain and at the mutated γ3 constant gene of the mutant *IgH* locus. (B) $CD19^+$ cells were sorted from the bone marrows (BM) of $Rag2^{-/-}$ and A150/A150 $Rag2^{-/-}$ mice, and quantitative RT-PCR was performed on total RNA. The *Gapdh* and *Ywhaz* transcripts were used for normalization. Iμ, μ0, and DSP transcript levels were quantified by

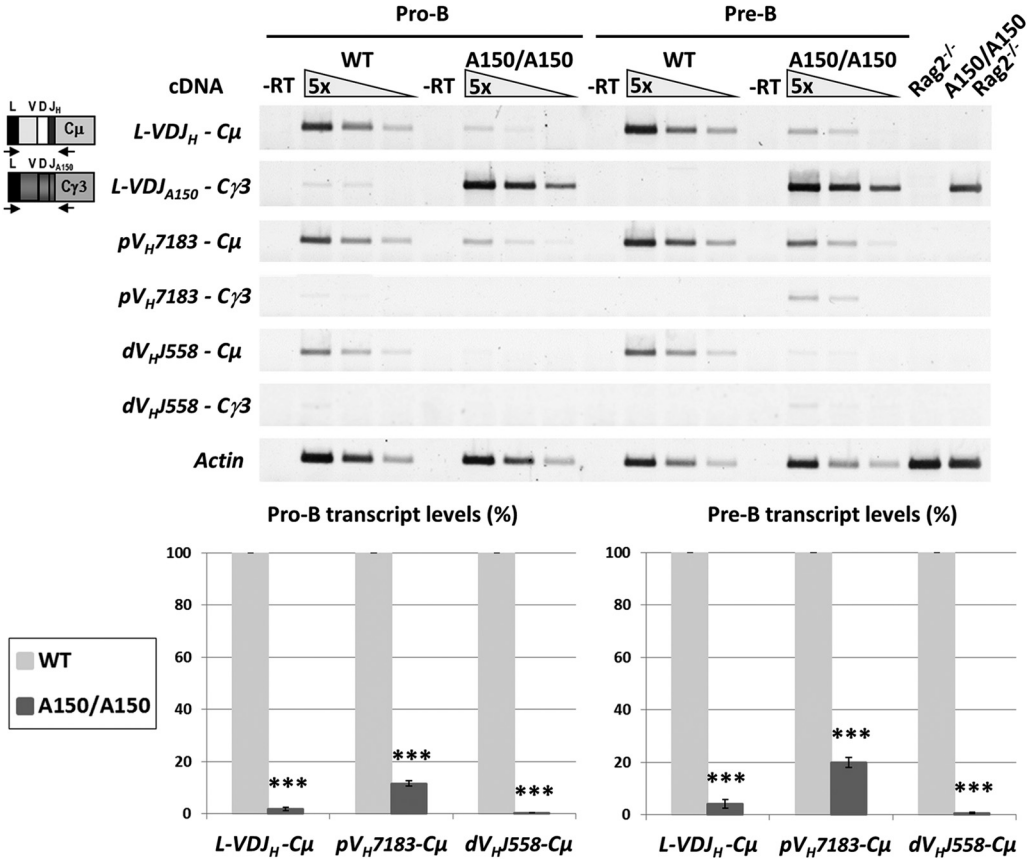

FIG 4 Analysis of μ and γ3 heavy chain transcripts in A150 pro-B and pre-B cells. Pro-B and pre-B cells were sorted from the bone marrows of WT and A150 mice, and RT-PCR was performed on total RNA. A specific primer that binds the leader sequence of the inserted V_H segment (schematics on the left), degenerate primers that bind the V_{H7183} or V_{HJ558} gene family, and specific Cμ1 or Cγ3-1 reverse primers were used to amplify the mature VDJ-Cμ and VDJ-Cγ3 transcripts. The actin transcripts were used for normalization of single-stranded DNA input. Single-stranded cDNAs from $Rag2^{-/-}$ and A150/A150 $Rag2^{-/-}$ mice were included as controls. The corresponding histograms are shown ($n = 2$ for each experiment, with a pool of >3 mice). ***, $P < 0.001$.

the mutant allele. Only in heterozygous mice was IgM detected, but in this context, IgM was produced by the wild-type allele in WT/A150 mice and by the B1-8 allele in B1-8/A150 mice.

In B1-8/A150 mice, in stark contrast to results for WT/A150 mice, the majority of B cells were subject to allelic inclusion, and the ratio of double producers to single producers was even higher in the spleen, suggesting that two different isotypes with allelic inclusion can drive B cell development and may confer a selective advantage over single producers. The possibility of B cell development under conditions of allelic inclusion of two different μ heavy chains was reported previously (34). Our findings extend this notion to different isotypes, with potentially different specificities.

Interestingly, we found that about 1/10 of WT/A150 bone marrow B cells were IgM single producers. This is more remarkable if one takes into account that only 1/3 of translational reading frames allow the production of μ heavy chains from the WT allele. How, then, are IgM-only-expressing B cells generated, while the γ3 heavy chain is presumably constitutively expressed? A mechanism such as an induction of apoptosis of B cells that coproduce IgM and IgG3 is unlikely; the phenotype of B1-8/A150 mice argues against it. Alternatively, signals transmitted through the μ pre-B cell receptor (pre-BCR) may silence *γ3* heavy chain gene expression on the alternate allele at the transcriptional level. This is unlikely, because of the biallelic nature of *IgH* gene transcription (39–41). Additionally, signaling through the pre-BCR downregulates the expression of surrogate light chains but not that of *IgH* genes (42). Finally, if the μ pre-BCR silenced *γ3* heavy chain gene expression at the transcriptional level, it would be apparent in B1-8/A150 mice, which is clearly not the case.

Instead, the data point to a deletional process. Several mechanisms can be invoked, including V(D)J recombination, V_H re-

setting the corresponding transcript levels in $Rag2^{-/-}$ controls as 100% ($n = 4$). (C) Analysis of germ line and coding transcripts at the ectopic transcription unit by semiquantitative RT-PCR. *Actin* transcripts were used for normalization ($n = 4$). (D) Schematic indicating the relative positions of the transcripts analyzed within the variable region. AST, antisense transcripts; ST, spliced transcripts (sense). (E) Analysis of proximal (pV_{H7183}) and distal (dV_{HJ558}) germ line transcripts by semiquantitative RT-PCR. Results of two independent experiments are shown. *Actin* transcripts were used for normalization ($n = 4$). S, spliced transcripts; US, unspliced (antisense/primary sense) transcripts. (F) Quantification of sense and antisense transcript levels as assayed by semiquantitative RT-PCR in panel E. (G) Quantification of intergenic antisense transcripts within the V_{HJ558} cluster and of PAX5-activated intergenic repeat 4 antisense transcripts by quantitative RT-PCR. The *Gapdh* and *Ywhaz* transcripts were used for normalization ($n = 4$). Data are presented as means ± SEM. **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

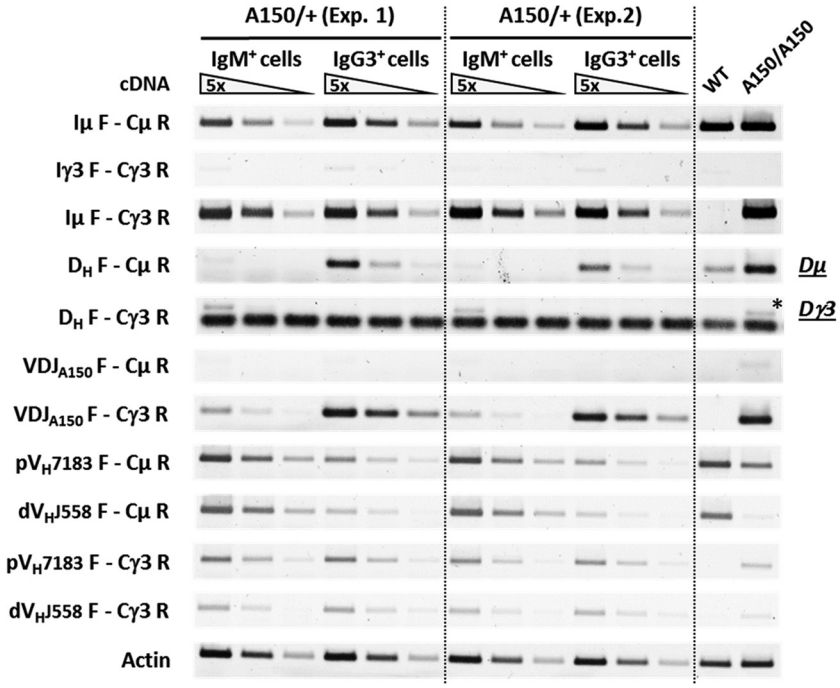

FIG 5 Transcription analysis upon ectopic V(D)J recombination in heterozygotes. IgM^+ and $IgG3^+$ populations were sorted from the bone marrows of A150/WT mice, and RT-PCR was performed on total RNA. Specific primers were used to amplify the indicated spliced transcripts. Dμ and Dγ3 transcripts, resulting from endogenous DJ_H and ectopic DJ_H recombination intermediates, respectively, are indicated. Controls included the corresponding transcripts from WT and A150/A150 mice. The actin transcripts were used for normalization of single-stranded DNA input. The data shown are the results of two independent sorting procedures and experiments (pools of 4 mice).

placement, and CSR. We found no evidence for V_H replacement, and only very low (if any) levels of CSR were detected, making it difficult to quantify its real contribution. In contrast, the vast majority of deletional events involved an ectopic V(D)J recombination. Thus, the inserted unit in the *IgH* constant region provides sufficient elements for chromatin opening, transcription, targeting, and activity of the RAG complex (43). This suggests that the ectopic J_H segments are brought into close proximity of endogenous D segments for ectopic V(D)J recombination to occur, implying that the endogenous and ectopic Eμ enhancers are positioned nearby (see below).

For WT/A150 mice, we cannot, at first glance, ascertain whether *cis* exclusion results from allelic exclusion or from a combination of both allelic exclusion and γ3 heavy chain-mediated signaling. In homozygotes, *cis* exclusion may also result from γ3 heavy chain-mediated feedback inhibition. However, we think that *cis* exclusion is likely to result from mechanisms that act in *cis*. Indeed, it would be difficult to figure out why (i) in heterozygous mice, γ3 heavy chain-mediated signaling would lead to such perfect exclusion of the μ allele precisely on the mutant chromosome but not on the WT allele; and (ii) in homozygous mice, none of the productive endogenous rearrangements [i.e., not involving Cμ deletion through ectopic V(D)J recombination] led to IgM expression. These remarks rather point to an effect which likely results from Eμ duplication and a potential perturbation of Eμ interactions.

How relevant is *cis* exclusion to genuine allelic exclusion? Remarkably, *cis* exclusion recapitulates on the very same allele the main known features of allelic exclusion regarding V_H gene family restriction. Indeed, various studies have shown that allelic exclusion of proximal V_H genes is less stringent than that of distal V_H genes, especially for the most proximal V_H genes (e.g., see references 19, 44, and 45). Consistent with these findings, we found that distal V_H-DJ_H recombination was virtually inhibited and proximal V_H-DJ_H recombination was only reduced. Moreover, the differential recombination of proximal versus distal V_H genes correlated well with the levels of their corresponding sense and antisense germ line transcripts. This correlation between germ line transcription and *cis* exclusion is also relevant to allelic exclusion. The developmentally regulated sense and antisense transcription of the V_H region occurs following D-J_H recombination, likely to provide accessible substrates for V_H-DJ_H recombination (35, 36). The correlation that we found between V_H recombination and germ line transcription during *cis* exclusion is consistent with a model in which sense and antisense transcription within the V_H region is downregulated by allelic exclusion, not by the recombination event itself (36). In support of this notion, distal V_H transcription was already inhibited on the *cis*-excluded allele in the absence of detectable distal V_H-DJ_H recombination. The latter finding strongly suggests that the control of germ line transcription is the primary event during allelic exclusion.

With regard to V_H germ line transcription, an important issue is to explain why sense and antisense transcription is affected within the V_H domain. It is plausible that, similar to findings for μ-transgenic mice (46), the development of A150 mutant B cells is accelerated such that there is not enough time for the opening of the variable region's chromatin. We cannot formally exclude this possibility, although we note that in A150 mice, D-J_H rearrangements occurred (with even a slight accumulation of DJ_H intermediates) at the right developmental stage (pro-B stage), in contrast

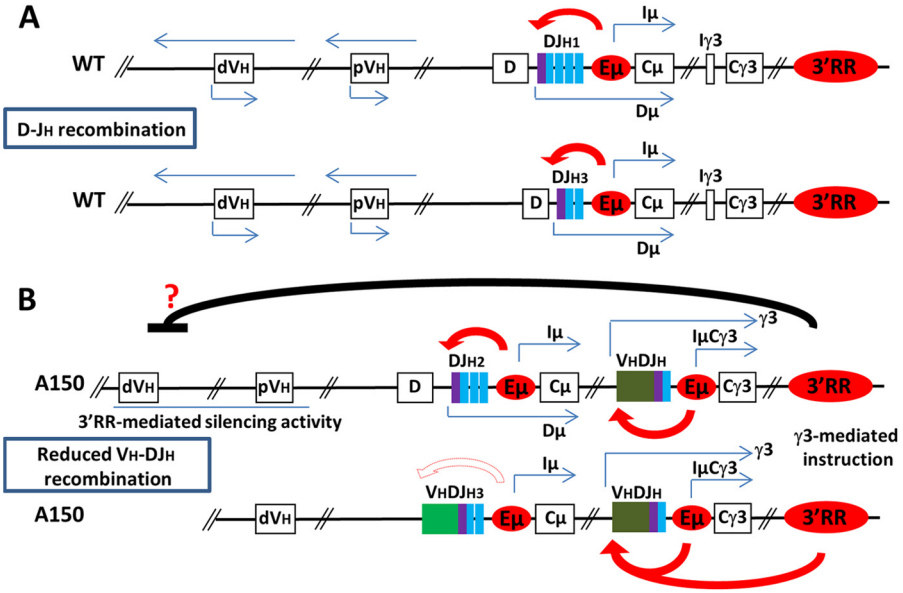

FIG 6 Model for the transcriptional control of allelic exclusion. The model shown represents a speculative view of how allelic exclusion is regulated through an interplay between the Eμ enhancer, the 3′RR, and signaling. For the sake of clarity, only the interactions between the Eμ enhancer and the 3′RR are highlighted. Other *cis*-regulatory elements (such as IGCR1) which also play an important role in allelic exclusion are not shown. (A) In this model, a productive rearrangement on one allele instructs the 3′RR on the second allele to mediate a transcriptional silencing activity within the V_H region, leading to downregulation of sense and antisense transcription and V_H-DJ_H recombination. (B) In A150 mice, the prerearranged VDJ gene in the constant region allows for premature production of the γ3 heavy chain, which instructs the 3′RR to mediate its silencing activity within the V_H region, leading to an impairment of V_H-DJ_H recombination. The 3′RR preferentially interacts with the proximal, ectopic Eμ enhancer to boost γ3 gene expression. In the absence of endogenous Eμ/3′RR cooperation, rearranged μ gene transcription is too low to allow robust signaling (see the text for details). pV_H, proximal V_H cluster; dV_H, distal V_H cluster.

to μ-transgenic mice, in which D-J_H rearrangements occurred mostly at the pre-B stage (46). We rather favor the view that impairment of V_H germ line transcription in A150 mice results from altered interactions between *cis*-regulatory elements. In this context, it was shown that distal V_H transcription was Eμ independent, while transcription of the most proximal V_H genes was affected by Eμ (11–14, 18, 47). Our interpretation is that these defects largely (but not exclusively) reflect a transcriptional silencing activity which is mediated by the other master *cis*-acting element of the *IgH* locus, namely, the 3′RR. This is consistent with our recent finding that in the absence of the 3′RR, germ line transcription is upregulated along the V_H domain and distal V_H-DJ_H recombination is enhanced, which led us to propose that a productive rearrangement on one allele instructs the 3′RR to inhibit germ line transcription within the V_H region on the second allele (F. Z. Braikia and A. A. Khamlichi, submitted for publication) (see the model in Fig. 6). Within the time window when the 3′RR mediates its silencing activity within the V_H domain, the Eμ enhancer mainly focuses on DJ_H transcription (14).

Thus, duplication of the Eμ enhancer appears not to affect the 3′RR-mediated silencing activity within the distal V_H domain. In contrast, cooperation between the endogenous and ectopic Eμ enhancers may explain the increased levels of Iμ and μ0 germ line transcripts, implying that the two enhancers lie in close proximity prior to D-J_H recombination. The occurrence of ectopic D-J_H recombination involving synapsis and ligation of an endogenous D segment and a remote ectopic J_H segment provides an indirect support of this interpretation. This is also consistent with the finding that the 3′RR is dispensable for germ line transcription within the D-Cμ domain (Braikia and Khamlichi, submitted).

Why is transcription of μ genes reduced on the *cis*-excluded allele? Our findings suggest that duplication of the Eμ enhancer does not necessarily lead to upregulation of μ gene expression. A likely explanation is that following V_H-DJ_H recombination, the 3′RR preferentially interacts with the most proximal, ectopic Eμ enhancer (Fig. 6). Interestingly, when the Iγ3 germ line promoter was replaced with the Iγ1 germ line promoter, transcription from ectopic Iγ1 but not from the endogenous 3′RR-proximal Iγ1 was reduced (31). Although this finding is indirect, as that study was performed on splenic B cells, it suggests that when upstream duplicated targets are available, the 3′RR prominently interacts with the most proximal target.

Significantly, any ectopic V(D)J recombination on the A150 allele deletes the endogenous Eμ enhancer. Ectopic Eμ, in cooperation with the 3′RR, would thus mediate a high-level expression of ectopically rearranged proximal V_H- and distal V_H-containing γ3 genes. The relatively higher levels of their corresponding transcripts, though they occur in only a fraction of heterozygous B cells, provide a physiological support of this model. Moreover, we recently showed that upon completion of V(D)J recombination, the 3′RR shifts from a transcriptional silencer to an enhancer of both Eμ- and P_{VH}-derived transcription (Braikia and Khamlichi, submitted). Thus, the Eμ enhancer likely requires the cooperation of the 3′RR for high-level expression of rearranged heavy chain genes, which would be essential for the strength of signaling required for the maintenance of allelic exclusion.

In conclusion, the *cis* exclusion reported here recapitulates essential features of allelic exclusion and thus provides important insights into the transcriptional and recombinational mecha-

nisms that underlie genuine allelic exclusion. Distal V_H-DJ_H recombination is virtually inhibited, while proximal V_H-DJ_H recombination is only reduced. This correlates perfectly with an inhibition of distal V_H transcription and a reduced proximal V_H transcription. The developmental stage-dependent dynamics of Eμ-3′RR interaction (though by no means only this) may play a critical role in allelic exclusion, in that, as instructed by a productive rearrangement, the 3′RR downregulates germ line transcription in the variable region of the excluded allele. On the productive allele, the 3′RR cooperates with the Eμ enhancer to achieve high expression levels to yield a robust signal for the maintenance of allelic exclusion. Thus, our study reveals that an intricate, developmental stage-dependent interplay between *cis*-acting elements and signaling plays an important role in the initiation and maintenance of allelic exclusion.

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N.P. and C.L. performed research; Z.O. and M.L.B. generated the A150 mouse line; M.M. managed the mouse lines; N.P., C.L., and A.A.K. analyzed data; and A.A.K. designed research and wrote the paper.

We declare that we have no conflicts of interest.

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